EXHIBIT H

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Chairman: Prof. Dr. Dr. h.c. mult. A. Mayr

The Genome of the Vaccinia Virus Ankara Strain and its Alteration during Attenuation

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Dean: University Professor Dr. J. Unsheim

Examiner: University Professor Dr. Dr. h.c. mult. A. Mayr

Co-examiner: University Professor Dr. M. Merkenschlager

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Dedicated to my dear parents

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1. Introduction

As a result of the global use of the vaccinia virus (orthopox virus commune), it was possible to declare human pox virus infections extinct in 1980. However, this does not extinguish interest in the vaccinia virus, the prototype of genus orthopox virus. This virus has recently returned to the focus of attention as a result of its ability to absorb and express foreign genes, as well as to induce an antibody response against expressed antigens in the body. Due to vaccination complications observed in the past following initial immunizations with various vaccinia virus strains, research is currently focused on examining genetically fixed virulence. The objective is to find virus strains that are free of side-effects and thus suitable for the construction of "safe" vaccinia recombinants.

The MVA strain, attenuated in more than 570 passages on chicken embryo fibroblast cell cultures, differs from other vaccinia virus strains in a number of ways, especially with respect to its significantly reduced virulence. Because of our extensive knowledge of its biological characteristics, this virus is a strong candidate to serve as a model in studies of virulence.

The purpose of the present study is to contribute to describing the changes in the virus genome occurring during the passage period and to assign characteristic phenotypic properties to these changes.

2. Literature

2.1 Genome structure of vaccinia viruses

The genome of vaccinia viruses, in all pox viruses, consists of a linear, double-stranded DNA molecule. The mean molecular weight has been reported as being 123 Megadalton (MD), corresponding to approximately 186,000 base pairs (bp) (Moss, 1985).

However, references in the literature vary considerably with regard to exact genome size. The longitudinal measurement of the DNA molecule under an electron microscope and examinations of the sedimentation behavior of viral DNA in viscosity gradients were long considered the most reliable methods of determining molecular weight (Geshelin and Berns, 1974; Gafford et al., 1978). More recently, the size of the Vaccinia genome has been measured following digestion of the DNA with restriction enzymes. In this process, the molecular weights of the individual restriction fragments are determined following gel electrophoretic separation through comparison with molecular weight standards and are then summed (Gangemi and Sharp, 1976; Müller et al., 1977). However, because the separation capacity of the agarose gels decreases substantially with increasing molecular weight (Gangemi and Sharp, 1976; McCarron et al., 1978), estimating the size of DNA fragments in the region of more than 25 kilobase pairs (Kbp) becomes problematic (Mackett and Archard, 1979).

Thus, for example, the molecular weight data for the largest HindIII fragments of the vaccinia virus strain Western Reserve (VV WR) range, depending on the research group, between 30 MD (≈ 45 Kbp) and 37 MD (≈ 56 Kbp), while total genome length ranges from 119 MD (≈ 199 KbP) to 132 MD (≈ 199 Kbp) (Gangemi and Sharp, 1976; McCarron et al., 1978; Mackett and Archard, 1979; Panicali et al., 1981; DeFilippes, 1982).

A unique feature of the vaccinia virus genome is the covalent bond of the two DNA strands at the genome end. Under denaturation conditions, the individual strands – or, following restriction digestion, the terminal fragments – remain connected to one another (Geshelin and Berns, 1974) and can quickly renature again ("snap back" mechanism). This is useful in the identification of the genome ends following restriction enzyme digestion (DeFilippes, 1976; Wittek et al., 1977; Jaureguiberry, 1977; Mackett and Archard, 1979).

In this process, only terminal fragments renature into double-stranded DNA molecules and, following electrophoresis, can be identified in a comparison with the fragment pattern of the total DNA. The remaining, denatured, single-stranded DNA is not visible in the gel as a band. The covalent bond of the genome ends, in this case, is not specific for vaccinia DNA. Similar DNA structures are suspected in the genome of the virus of African swinepox (genus iridoviridae) (Ortin et al., 1979), and are the basis for existing replication models in adenoviruses and parvoviruses (Moss, 1985).

Another unique aspect of orthopox DNA are the "Inverted Terminal Repeat" (ITR) regions in the vicinity of the genome ends. In this connection, base sequences of the left terminal at the right end of the genome repeat themselves, but are conversely complementary to one another (Wittek et al., 1977; Wittek et al., 1978a). The ITR range of VV WR, with a magnitude of 10,500 base pairs, is especially well-researched (Garon et al., 1978). In this region, Wittek and Moss (1980) found two blocks of sequence repetitions near the genome end, in which a repetition of 70 base pairs occurs 13 or 17 consecutive times. The examination of various virus plaques of the VV WR revealed substantial variation in number and size of the 70 bp sequence repetitions (Baroudy and Moss, 1982). ITR regions apparently occur in the genomes of all orthopox viruses, with the exception of variola virus strains. The underlying sequences are similar, leading to cross-hybridization, both within the genome and across species (Mackett and Archard, 1979).

Because of their incomplete base pairing, the first 104 nucleotides at both genome ends form an inverse complementary hairpin structure known as "flip-flop terminal loops." The currently valid replication models for vaccinia virus DNA are predicated on the existence of this hairpin structure (Baroudy et al., 1982, 1983). When the "terminal loop" is unfolded, individual strand fractures in the DNA of the ITR region can divide the virus genome into linear individual strands of DNA and free 3'OH ends as starters for viral DNA polymerase ("self-priming"). It is believed that the replication continues in the form of ITR sequences becoming attached to one another (Wittek and Moss, 1980; Baroudy et al., 1982), leading to the occurrence of the transitional concatemer of the viral DNA observed during replication (Moyer and Graves, 1981). Merchlinsky et al. (1988) were able to identify cloned replication concatemers of the vaccinia DNA as double copies of the terminal hairpin structures. At the same time, they localized, at their edges, interfaces of a vaccinia nuclease that can produce individual strand fractures and close them again.

The examination of the genome of various orthopox species with restriction endonucleases produced numerous constantly wandering fragments, all of which can be assigned to the central portion of the genome. Especially the restriction enzyme HindIII proved suitable for physical mapping (Mackett and Archard, 1979; DeFilippes, 1982; Esposito and Knight, 1985). The gene maps show a highly uniform distribution of the enzyme interfaces in the inner genome region. About two-thirds of the total genome are highly conserved in all orthopox species. Variations occur in the length and structure of the terminal fragments, and genome lengths can fluctuate considerably. Esposito and Knight (1985) calculated the genome sizes of cowpox virus strains at more than 220 Kbp, while the size of variola virus strains amounted to only 180 Kbp. Even within an orthopox virus species, it is possible to differentiate among individual isolates on the basis of the structure of their terminal fragments.

2.2 Occurrence of genome changes

Length variations in the genome of orthopox viruses and their localization in the terminal genome region are principally associated with the mechanism of DNA replication and the special structure of the genome ends.

Thus, for example, the examination of a phenotypically uniform vaccinia virus population with restriction enzymes revealed differences in the nature of the corresponding DNA fragments, which were discernible in the appearance of unclear or submolar bands in the agarose gel and could be eliminated by cloning the virus population (Wittek et al., 1978b). In this context, Moss et al. (1981a) described a highly variable, unstable replication of short "Tandem repeat" sequences in the ITR region of the genome of randomly selected virus plaques. McFadden and Dales (1979) reported, in connection with the study of temperature-sensitive vaccinia virus mutants, on mirror image-like microdeletions in the genome end in 20% of all virus clones.

In replication models for vaccinia viruses (Baroudy et al., 1982; 1983; Wittek and Moss, 1980), the ITR regions of the virus genome play an important initiator role in DNA replication. The placement of the ITR sequences adjacent to one another in the replication concatemer serves as a starting point for the synthesis of a DNA daughter strand. If this placement occurs unevenly, shifts and duplications of the repetitive sequences can occur, resulting in the variability of the genome ends. In this context, the conservation of a symmetrical genome structure can be viewed as a fundamental principle (Wittek, 1982). Thus, for example, virus mutants are known in rabbitpox viruses, in monkeypox viruses and in cowpox viruses, in which segments of one genome end are replaced by duplicates of the sequences of the opposing genome end (Moyer et al., 1980; Esposito et al., 1981; Pickup et al., 1984). If corresponding translocations occur, the genome size of the mutants can increase by up to 30 Kbp, despite the fact the sequences have in fact been lost.

However, genome changes also occur in the constant genome region, and can be associated with the loss of individual nucleotides or the deletion of entire sequence segments. Point mutations in the genome usually occur as a result of adjustment to suddenly occurring selection conditions, such as the use of antiviral agents or the selective increase in the ambient temperature (Tartaglia and Paoletti, 1985; Drillien and Spehner, 1983). In contrast, virus mutants exhibiting substantial deletions in their genome have been isolated following permanent passages in cell cultures. Two such mutants of the VV WR had lost a piece of DNA larger than 10 Kbp in the left half of the genome immediately following the ITR region (Moss et al., 1981b; Panicali et al., 1981). Although the sequences verifiably coded for several proteins, there were no recognizable differences in comparison to the wild types with regard to growth in the cell culture.

Another method for the development of new genotypes in orthopox viruses – recombination – has been known for some time (Woodroofe and Fenner, 1960). When a cell culture is simultaneously infected with different virus strains, an exchange of genetic material occurs during replication in the host cell, due to the "molecular intersection" of adjacent DNA strands. The conformity between large genome segments of the orthopox viruses not only means that recombinants of a virus species are formed, but also facilitates the development of recombinants among different species of orthopox viruses. Hybrids produced from cowpox viruses and variola viruses exhibited the biological characteristics of cowpox viruses and those of variola viruses (Bedson and Dumbell, 1964b). A total of ten phenotypically different recombinants were isolated in these experiments. Bedson and Dumbell (1964a) also described the unexpectedly fluctuating pathogenicity of such species variants for various hosts. They recombined rabbitpox viruses (pathogenic for rabbits and mice) and alastrim viruses (apathogenic for rabbits and mice) into hybrids, some of which were only pathogenic for rabbits, while others were only pathogenic for mice. The biological and genetic characterization of recombinants between vaccinia viruses and mousepox viruses showed that the pathogenic characteristics of different virus species can also accumulate (Chernos et al., 1985). The natural occurrence of new pox virus isolates with previously unknown phenotypes could be attributable to the genetic recombination of original virus species. Perhaps this could even resolve the question of the origins of the vaccinia virus (Gershon et al., 1989).

2.3 Localization of genome changes

Insights into the structure and function of a virus genome are only possible when changes in the genetic material affect the phenotypic characteristics.

The examination of virus mutants that lacked a specific, identifiable characteristic – due to either point mutations or deletions – proved to be especially suitable. For example, Gemmel and Fenner (1960) isolated rabbitpox virus mutants with atypical growth on the chorioallantois membrane. In other laboratories, temperature-sensitive, host cell-dependent or agent-resistant vaccinia virus mutants were selected by means of direct mutagenesis (Fenner and Sambrook, 1966; Sambrook et al., 1966; Subak-Sharpe et al., 1969; Dubbs and Kit, 1964).

The analysis of these mutants in complex recombinant experiments and initial genome studies involving restriction enzymes were then able to provide information

on these loci responsible for the development of characteristics (Moyer and Rother, 1980; Drillien et al., 1982).

The development of marker rescue technology (Hutchison and Edgell, 1971), that is, the targeted recovery of a genetically anchored characteristic, made it possible to precisely localize genes in the virus genome. In the cell culture mixture, the genome of the wild type virus, cut with restriction enzymes, is added to the infectious DNA of a virus mutant with loss of characteristics. During virus replication, parts of the DNA of the mutant genome recombine in the cell with complementary transfixed wild type virus fragments. If, in this process, the deletions of the mutants are bridged by the attached DNA fragments, the resulting virus recombinant will regain the lost, phenotypic markers. Through the fluctuating use of various DNA fragments in experiments, one determines which fragment reestablishes the original characteristic. This fragment is then further sub-cloned, using other restriction enzymes, so that the resulting sub-fragments can be used to repeat the marker rescue experiments. In this manner, the size of the deletion in the genome of the virus mutant can be determined with great precision. Once the gene region coding for the development of characteristics has been narrowed down as much as possible, the gene sequence is analyzed. Using the identification of transcribed sequence segments and the decoding of the resulting amino acid sequence, homologies with already registered gene sequences can be determined and initial structure-effect relationships among the gene products can be derived.

Soon after its development, marker rescue technology was already being used to successfully localize mutations in adeno and herpes viruses (Arrand, 1978; Frost and Williams, 1978; Bookout et al., 1978; Knipe et al., 1978). In the case of pox viruses, however, the lack of infectiousness of the isolated virus DNA initially stood in the way of the application of this method (Sam and Dumbell, 1981). The key alteration was to incorporate the DNA fragments required for "rescue" into infected cells by means of transfection in the surplus. Only the use of a suitable search system (screening) allows for the selection of the desired virus recombinants. The first successful market rescue experiments with vaccinia viruses were conducted by Sam and Dumbell (1981), on the characterization

of temperature-sensitive virus mutants, and by Nakano et al., (1981), on the methodology of DNA transfection to deletion mutants. This new technology substantially accelerated progress in gene mapping for vaccinia viruses.

Meanwhile, an entire palette of various temperature-sensitive vaccinia virus mutants were genetically characterized (Condit et al., 1983; Drillien and Spehner, 1983). In this process, it became evident that the responsible gene defects occurred in distributed form across the entire central region of the genome, but not at the genome ends. For this reason, it is assumed that the majority of the genes that code essential enzymes and regulator proteins, for the purpose of viral replication under in-vitro conditions, lie in the highly conserved central region of the vaccinia virus genome.

The thymidine kinase (TK) enzyme is the first vaccinia virus protein whose gene in the virus genome could be precisely localized with the aid of marker rescue (Weir et al., 1982). TK-negative virus mutants were reproduced by transfection with wild type virus DNA fragments of the TK-positive phenotype. A plaque test on TK-negative cells was used for selection purposes. The simple selection system made this gene locus, located in the HindIII J-fragment, the preferred insertion point for foreign genes.

Jones and Moss (1984) described the mapping of the gene of vaccinia DNA polymerase. To this end, they used a mutant which is resistant to DNA polymerase inhibitor phosphono-acetate (PAA). In transfection experiments, it was possible to demonstrate that only a HindIII fragment (E) of the resistant virus mutant, located in the left half of the genome, recombines with the DNA of a PAA-sensitive wild type virus to form the PAA-resistant phenotype.

Tartaglia and Paoletti (1985) found the genetic locus for resistance to the antiviral inhibitor rifampicin in the HindIII D-fragment of the vaccinia virus genome. This agent prevents the combination of already pre-synthesized proteins into mature, infectious particles. A sequence analysis of the DNA of rifampicin-resistant wild types showed that only one point mutation in the coding region for a 62 KD polypeptide is sufficient for the development of resistance (Tartaglia et al., 1986; Baldick and Moss, 1987).

Marker rescue experiments by Villarreal and Hruby (1986) localized the gene for a different agent resistance, resistance against α-amanitine in the HindIII N-fragment. An examination of the responsible gene sequence is intended to provide new insights into the function of soluble virus proteins during transcription processes in the nucleus of the host cell (Tamin et al., 1988).

Shida (1986) studied the structure of vaccinia hemagglutinin (HA) and its locus. In transfection experiments with HA-negative mutants of the vaccinia virus strain IHD-J, the coding region was mapped on the outermost right end of the HindIII A-fragment. In this connection, the red coloration of HA-positive virus plaques by chicken erythrocytes served as a selection system. Flexner et al. (1987) accomplished a noticeable attenuation with vaccinia viruses by the targeted deletion of the hemagglutinin gene. Because the hemagglutinin gene is not essential for virus replication and recombinants can be easily selected, the locus is well-suited as an insertion point for foreign DNA.

Perkus et al. (1986) used the marker rescue method to construct insertion mutants with vaccinia viruses. By inserting the thymidine kinase gene of the herpes simplex virus into the vaccinia virus genome, they were able to characterize a number of loci where inserted foreign DNA is expressed without impairing the replication ability of the carrier viruses.

Paez et al. (1985) isolated variants of the vaccinia virus strain Western Reserve with characteristic, small plaques. The structural change in the gene of a 14 KD shell protein from the HindIII A-fragment was responsible for the "small" plaque phenotype (Dallo et al., 1987). Rodriguez and Esteban (1989) recommend the plaque phenotype as a selection marker for the construction of vaccinia recombinants.

Cowpox viruses (KPV) produce noticeably hemorrhagic pox lesions on scarified skin segments of rabbits and on the chorioallantois membrane of infected chicken eggs; so-called "white" virus mutants lack these phenotypic characteristic. Pickup et al. (1984) found large deletions at the right end of the genome of such mutants. In marker rescue experiments, it was possible to localize the corresponding gene in the KpnI D-fragment of the KPV genome.

It codes for the amino acid sequence of a 38 KD protein, which is very similar to plasma proteins which, as inhibitors of various serine proteases, intervene in the regulation of blood clotting. The viral protein presumably possesses similar biological activity, thus causing hemorrhage of the pox lesions (Pickup et al., 1986).

In infected cells, cowpox viruses induce large cytoplasmatic type A inclusion bodies, the appearance of which is generally used as a morphological characteristic to differentiate between cowpox viruses and vaccinia and/or variola viruses (Ichihashi et al., 1971). The type A inclusion bodies consist of a viral "late" 160 KD polypeptide produced in the surplus (Shida et al., 1977a,b). Vaccinia viruses produce an antigenetically closely related 94 KD polypeptide instead of the 160 KD protein, but do not form any type A inclusion bodies (Patel et al., 1986). Through transfection of KPV DNA in the genome of vaccinia viruses, Funahashi et al. (1988) were able to find the gene of the 160 KD protein in the HindIII A-fragment of the KPV genome. In hybridization experiments, a comparable, complementary gene structure was localized in the HindIII A-fragment of the vaccinia genome. In expression experiments (cat assay) involving the promoter region of the KPV gene, a substantial increase in activity was achieved in comparison to other vaccinia promoters. These results suggest that the gene locus may serve as a possible insertion point for foreign genes, although the structure and function of the actual vaccinia gene require further investigation.

2.4 Genome change and virulence

In light of renewed application as a vaccine virus, the investigation of the genetic fundamentals of virulence characteristics of vaccinia viruses is very significant.

The ability to form thymidine kinase (TK^+) plays a special role in determining the extent of virulence in vaccinia viruses. Buller et al. (1985) reported that vaccinia virus vector recombinants with a TK-negative phenotype for mice, following intraperitoneal and intracerebral administration, were significantly less virulent than the TK-positive vaccinia virus wild types. The TK-negative phenotype, but not the foreign DNA inserted at the TK locus, was clearly responsible for the loss of virulence. Other vaccination

experiments with TK-negative vaccinia virus recombinants in rabbits and chimpanzees confirm these results (Buller and Moss, 1985).

Through permanent passages in persistently infected Friend Erythroleukemia (FEL) cells, it was possible to isolate various vaccinia virus deletion mutants that were characterized by small plaques. In addition to an 8 MD deletion at the left end of the genome (Paez et al., 1985), it was possible to demonstrate, using marker rescue experiments, that the plaque phenotype, independently of the large deletion at the left end of the genome, is attributable to the loss of a gene in the HindIII A-fragment, that is, in the highly conserved genome region. The gene codes for a 14 KD vaccinia virus shell protein, which appears to play an important role in virus penetration into the host cell (Dallo et al., 1987; Rodriguez and Esteban, 1987). In infection experiments with mice, Dallo and Esteban (1987) demonstrated that both genome changes cause an attenuation of the wild type virus. However, the 8 MD deletion in the left genome term causes greater loss of virulence than the deletion of the shell protein gene (Rodriguez et al., 1989).

Moss et al. (1981b) isolated a vaccinia virus mutant (6/2) with a large deletion in the left half of the genome, in the region of the HindIII fragments C/W. Transcription experiments showed that the mutants can no longer synthesize an entire group of RMS molecules. Although the missing genes are not required for virus replication in the cell cultures (there were no growth differences between the virus mutants and the wild type virus), comparative animal studies revealed a significant attenuation of the deletion mutant 6/2 (Buller et al., 1985). Precise sequence tests confirmed that a portion of these non-essential genes codes for secretory virus proteins known as virokines (Kotwal and Moss, 1988a,b). One of the identifying proteins, a 35 KD polypeptide, bears considerable similarity to a human protein that binds the complement 4B. The virus protein, in cleaned form, inhibits the classic complement cascade and could thus counteract the defense mechanism of the host organism. The absence of a second secretory, 12 KD vaccinia protein, the function of which is as yet unknown, is also involved in the reduced virulence of the

virus mutant 6/2 (Kotwal and Moss 1988b, Kotwal et al., 1989).

In both ITR regions of their genome, vaccinia viruses possess a gene that codes for another secretory virus protein, Vaccinia Growth Factor, or VGF (Twardzik et al., 1985). This 19 KD polypeptide can bond to the EGF (Epidermal Growth Factor) receptor of non-infected cells and stimulate their growth. Through bilateral deletion of the VGF gene, Buller et al. (1988a,b) were able to prevent virus-induced cell proliferation and weaken virulence in the mouse and in the rabbit. In contrast, there were no discernible differences between the constructed deletion mutants and the wild type virus in terms of their replication ability in cell cultures.

Another vaccinia virus variant was selected on the basis of its plaque morphology following direct mutagenesis with nitrous acid (Drillien et al., 1981). The host range of the virus mutant was significantly restricted. It could not be replicated on most human cell lines, and could only be poorly replicated on monkey cell lines. The host range mutants and the wild type virus do not differ in terms of growth on HEF cells, on a hamster cell line and on two mouse cell lines. The restriction enzyme analysis of the DNA showed that an approx. 18 Kbp DNA segment, including a portion of the ITR region, was deleted on the left side of the genome. The gene region responsible for the host range was increasingly narrowed in marker rescue experiments by deleting overreaching wild type virus DNA fragments. It was finally possible to localize, at the transition between the HindIII fragments M and K of the virus genome, a short DNA sequence that was sufficient to return the replication capacity on the human cell line HEP 2 to the host range mutants (Gillard et al., 1985). The results of sequencing and in-vitro translation of the genome region support gene expression. The host range gene codes for a 29 KD "early" polypeptide, which could contribute to maintaining protein synthesis in infected cells or counteract antiviral activity in certain cells. Because of its position in the vaccinia genome, the host range gene could be another example of a vaccinia gene which, in its direct relationship to host genes, intervenes in the regulatory mechanisms of the host organism. This serves as the basis for a presumed